Translation of Japanese Patent Laid-Open No. 9992/1996, WO9904815A1

METHOD OF RECOVERING PROTEIN

What is claimed is:

1. A method of recovering protein for separating and recovering protein from a liquid in which microorganisms or animal cells float, wherein said liquid is filtered by a microfiltration device, the protein-containing filtrate from said microfiltration device is filtered by an ultrafiltration device for enrichment, and filtrate from said ultrafiltration device is added to the feed stream to said microfiltration device.

2. The method of recovering protein according to Claim 1, wherein said ultrafiltration device is designed to have a membrane area in such a way that the filtrate from said ultrafiltration device flows at a rate equal to or higher than that for the filtrate from said microfiltration device.

3. The method of recovering protein according to Claim 1 or 2, wherein the filtrate from said ultrafiltration device is added to the feed stream to said microfiltration device after being irradiated with ultraviolet ray for sterilization.

Translation of the disclosure of the invention and the claims

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Disclosure of the Invention

The present inventors have carried out an intensive investigation for mechanism of a cholesterol-lowering action of the above-mentioned YM-16638 and have firstly found in the present invention that the said compound has an activating action for PPAR δ and γ. From such a finding and also from the reports up to now that thiazolidinedione-type compounds which are ligands of PPAR γ do not lower the serum cholesterol level, we suspect that PPAR δ may mainly participate in the above cholesterol-lowering action and made further studies for compounds having a PPAR δ activating action. Thus, an investigation has been carried out for the compounds having a PPAR δ activating action using a method characterized in measuring the PPAR δ activating action or the PPAR δ and γ activating action. As a result, it has been found in the experiments using higher animals that p-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)propoxy]phenylacetic acid disclosed in the Czech Patent CZ 281130 as a compound showing anti-inflammatory and anti-asthma actions has a PPAR δ and γ activating action and that, unexpectedly, the said

compound shows excellent serum cholesterol lowering action and LDL-cholesterol lowering actions as same as YM-16638 and, on the basis of such findings, the present invention has been achieved.

Thus, the present invention relates to a pharmaceutical composition having a cholesterol-lowering action which contains a compound having a PPAR δ activating action or a PPAR δ and γ activating action or a pharmaceutically acceptable salt thereof as an effective ingredient.

The present invention further relates to a pharmaceutical composition where the above cholesterol-lowering action is an LDL-cholesterol-lowering action.

The present invention still further relates to a pharmaceutical composition having a cholesterol-lowering action which contains p-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)propoxy]phenylacetic acid or a pharmaceutically acceptable salt thereof as an effective ingredient.

The present invention furthermore relates to a method for identifying a compound having a cholesterol-lowering action which is characterized in measuring the PPAR δ activating action or the PPAR δ and γ activating action.

The present invention will now be illustrated in detail as hereunder.

The term "a peroxisome proliferating agent activating

receptor PPAR activating action" used in the present invention means all of the actions in the initial stage in which the compound directly bonds to and acts on the ligand-bonding site of the receptor or indirectly acts thereon and expression of the function is resulted by the said ligand-bonded receptor. When it is judged that there is a statistic significance in the comparison of measured value determined by measuring the said receptor activating action with the activity value in the cells to which the compound is not added (here, dimethyl sulfoxide used as a solvent is added), it is concluded that "an activating action is available".

In the present invention, "cholesterol-lowering action" means an action where the cholesterol value in serum of a pathologic level (usually 220 mg or more which requires the therapy) is significantly lowered and a pharmaceutical composition showing such a cholesterol lowering action is useful for prevention and therapy of various diseases caused by an increase in serum cholesterol.

"Compound having a PPAR δ activating action or a PPAR δ and γ activating action" contained as an effective ingredient in the pharmaceutical composition having a cholesterol lowering-action in the present invention covers all of the compounds, both known and novel, having a PPAR δ activating action or a PPAR δ and γ activating action which are selected from various known compounds registered in the Chemical File

by means of an identifying method for the identification of the compound having a cholesterol-lowering action of the present invention and newly synthesized compounds by conversion of substituent(s) utilizing the mother nucleus of such selected compounds.

"Pharmaceutically acceptable salt" means a salt which is formed by the said compound with an acid or a base and is nontoxic to living body.

To be specific, it is an acid addition salt with inorganic acid or with organic acid or a salt with inorganic or organic base and specific examples of such a pharmaceutically acceptable salt are addition salts with mineral acid such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, nitric acid or phosphoric acid, organic acid such as formic acid, acetic acid, propionic acid, oxalic acid, malonic acid, succinic acid, fumaric acid, maleic acid, lactic acid, malic acid, tartaric acid, citric acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid or toluenesulfonic acid or acidic amino acid such as aspartic acid or glutamic acid; salts with inorganic base such as sodium, potassium, magnesium, calcium, aluminum or lithium, organic base such as methylamine, ethylamine or ethanolamine or basic amino acid such as lysine or ornithine; and ammonium salt.

Further, the compound of the present invention may form a hydrate, a solvate with ethanol, etc. or polymorphism and

the present invention covers all of such a separated hydrate, solvent or polymorphism and a mixed compound thereof.

A method for the identification of the compound having a cholesterol-lowering action according to the present invention is a method which is characterized in measuring a peroxisome proliferating agent activation receptor PPAR δ activating action or PPAR δ and γ activating action and it provides a method for the confirmation of a PPAR δ or a PPAR δ and γ activating action of the compound and for the selection of a compound having a cholesterol-lowering action. The said method comprises the steps of (a) preparation of a construction of expression cassette coding for functional fragment of PPAR δ or PPAR γ receptor, (b) preparation of a construction where one or more response element (s) for functional protein fragment bonding to the above receptor fragment and reporter gene are bonded, (c) co-transfection to host using the construction, (d) addition of the compound to be tested, (e) measurement of expression of the reporter gene and (f) selection of a compound showing a PPAR δ activating action or a PPAR δ and γ activating action by comparing the test compound with a control and, by the use of the said method, many compounds are able to be quickly and efficiently measured and randomly screened.

The above-mentioned steps (a) and (b) have been established in recent years as a ligand evaluating system for nuclear receptor and, to be more specific, it is a reporter

system utilizing a bond of the expression regulating factor of GAL 4, GAL 1 (galactokinase), GAL 7 (α-D-galactose-1-phosphate uridyltransferase) and GAL 10 (uridine diphosphoglucose-4-epimerase) which are galactose metabolic enzyme proteins expressed in an enzyme (*Saccharomyces cervisiae*) with its responsive element UAS_G (galactose upstream activating domain) (*Cell*, 40, pages 767-774, 1985; 52, pages 161-167, 1988; 52, pages 169-178, 1988; and 54, pages 199-207, 1988). Besides the reporter system utilizing the DNA-binding ability of GAL4 protein of enzyme mentioned in Example 1 which will be given later (*J. Biol. Chem.*, 270(22), pages 12953-12956, 1995), it is also possible to utilize a reporter system utilizing a responsive region (peroxisome proliferator responsive element; PPRE) to which DNA bonding domain of PPAR is bonded (*Proc. Natl. Acad. Sci. USA*, 94, pages 4312-4317, 1997; 91, pages 7355-7359, 1994; and *J. Biol. Chem.*, 268(8), pages 5530-5534, 1993) and a reporter system utilizing a bacteria tetracycline operon (*J. Biol. Chem.*, 270(41), pages 23975-23983, 1995).

With regard to the fundamental technique concerning a genetic manipulation necessary for constitution of vector mentioned in Example 1, it is possible to carry out that by referring to *Basic Methods in Molecular Biology*, 2nd Edition (Leonard G. Davis, W. Michael Kuehl, James F. Battey; Prentice-Hall International Inc., 1994) and *Saibo Kogaku* (Extra

Number) "Illustrated Bio-Experiments; (2) Fundamentals of Genetic Analysis" [by Hiroki Nakayama and Takahito Nishikata (published by Shujunsha) 1995].

The gene-expressing vector used in the step (a) of the present invention is pGBT9 DNA-Binding Domain Vector (vector size: 5.5 kb; having GAL4-DBD domain and ampicillin-resistant gene Amp^R sequence in the vector; manufactured by Clontec) which is a commercially available vector already containing the gene coding for the DNA-binding domain (GAL 4-DBD) of GAL 4 protein and, when a ligand binding domain of the desired nuclear receptor is introduced into a multiple cloning site (MCS) near the GAL 4-DBD domain, chimera protein expressed in the cell is well convenient being able to be a sensor in the present identifying method and, in place of pGBT 9, the product pAS2 DNA-Binding Domain Vector (manufactured by Clontec) can be used or the constitution is also possible by utilizing a method mentioned in the literature (*Cell*, 52, pages 169-178, 1988) or a modified method thereof.

A fused vector of reporter gene with responsive element to activated chimera protein used in the step (b) is prepared by constructing a known responsive element followed by introducing into expression vector containing commercially available luciferase (Picagene Vector 2 [PGV-B2]; manufactured by Toyo Ink) and the method therefor is that, usually, insertion is conducted via a short fragment having an appropriate

restriction enzyme site. Thus, for example, in order to integrate near a reporter gene, a common method where a reporter gene having an appropriate restriction enzyme, for example, is cut and then a responsive element is inserted is carried out and construction of the responsive element having an appropriate element to be used is synthesized on a DNA synthesizer.

Although the known responsive element can be prepared by a DNA synthesizer as well, it is also possible to use a substance which is already integrated into a commercially available suitable vector such as pG5CAT reporter plasmid (manufactured by Clontec) and it is preferred that a responsive element having a good responding property is selected from the host which will be mentioned later used for identification of compounds and is used.

Appropriate promoter and terminator sequences are preferably selected so as to be active in the host which will be mentioned later and have been well known in the related art. They are able to be bonded to structural gene and, optionally, marker group and other convenient elements by a standard technique in genetic engineering.

Examples of the preferred host cells used for expression of constitutions prepared in the step (c) mentioned in the identifying method for the compound having a cholesterol-lowering action according to the present invention

are bacteria, fungi and cells of insects or mammals where the representative ones are HepG2 cells, NIH-3T3, COS-1, COS-7, U-937, CV-1 and KI-293 and the particularly preferred ones are HepG2, CV-1 and NIH-3T3 cells. Preferably, the experimental condition for introduction of gene using those cells is a condition where cytotoxic property is little and amount of introduced gene is much and is hardly decomposed and the condition is not limited to a method for introduction of gene by lipofectamine utilized in Example 1 which will be mentioned later.

The step (d) is that a known compound registered in the Chemical File or a newly synthesized compound is diluted to an appropriate diluting rate and added to a medium of the cell to which gene was introduced already whereby the measurement of the next step (e) is carried out and, for example, that can be treated as a high through put screening system using a 96-well plate.

In the step (d), the compound is added to a medium of the cell under a condition of being dissolved in an appropriate solvent. Its incubation method is carried out under such a condition that two genes introduced into the cell and a substance acting thereon are necessary and sufficient for the final measurement as a reporter activity and, if the compound is not able to pass through the cell membrane, it is also possible that an appropriate single substance is added or a system having

no cell is used.

With regard to expression of reporter gene in the present invention, it can be measured in a transcription level or a translation level such as produced protein, enzymatic activity or proliferated amount of cell.

With regard to an index which is an object of the measurement in the step (e), an appropriate reporter gene has been well known in the related art. In addition to firefly luciferase (luc, PGV; manufactured by Picagene), its examples are sea pansy luciferase (luc, pRL; manufactured by Picagene), bacterial hybrid luciferase (lu × AB), chloramphenicol acetyltransferase (CAT) and β-D-galactosidase (lac Z).

In the step (f), a solvent for the test compound is used as a control, an index for the reporter gene expression of the step (e) is measured by the cell which is treated with the solvent only and, taking its activity value (control value) as 1.0, the relative ligand activity of the test compound is calculated. Compounds which show a significant activating action to PPAR δ or PPAR δ and γ are selected.

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Claims

1. A pharmaceutical composition having a cholesterol lowering-action which contains a compound having a peroxisome

proliferating agent activating receptor PPAR δ activating action or a PPAR δ and γ activating action or a pharmaceutically acceptable salt thereof as an effective ingredient.

2. The pharmaceutical composition according to claim 1, wherein the cholesterol-lowering action is an LDL-cholesterol lowering action.

3. A pharmaceutical composition having a cholesterol-lowering action which contains p-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)propoxy]phenylacetic acid or a pharmaceutically acceptable salt thereof as an effective ingredient.

4. A method for identifying a compound having a cholesterol-lowering action which is characterized in measuring a peroxisome proliferating agent activating receptor PPAR δ activating action or a PPAR δ and γ activating action.